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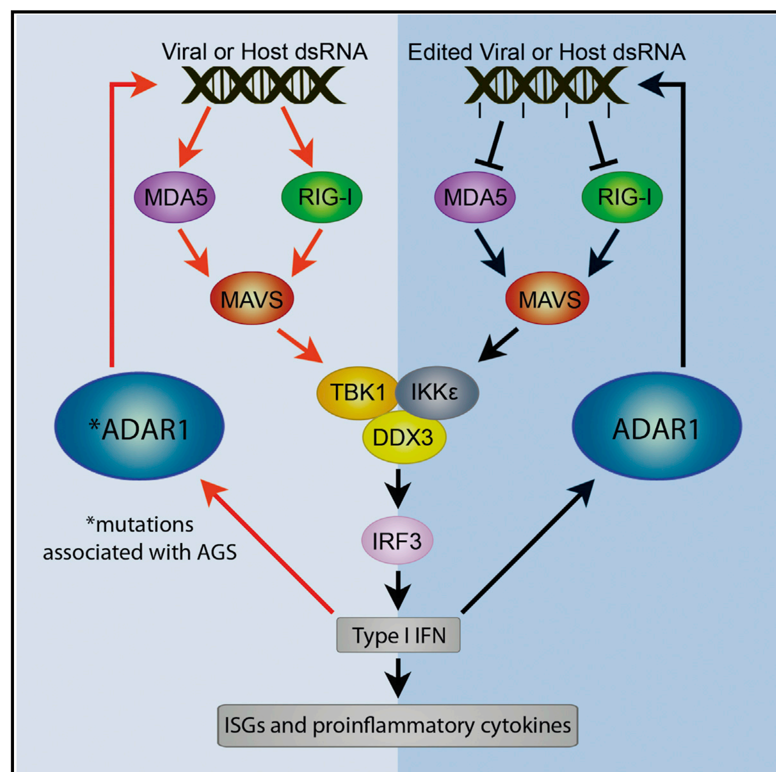
Graphical Abstract



Authors

Niamh M. Mannion, Sam M. Greenwood, ..., Liam P. Keegan, Mary A. O'Connell

Correspondence

liam.keegan@igmm.ed.ac.uk (L.P.K.),
mary.oconnell@igmm.ed.ac.uk (M.A.O.)

In Brief

Mice lacking *Adar1* have a heightened immune response and stress-related apoptosis. Mannion et al. demonstrate that this mutation can be rescued to birth by generating a double mutant with *Mavs*, an innate immune gene, indicating the central role ADAR1 plays in innate immunity.

Highlights

Adar1 mutant mouse embryonic lethality is rescued in *Adar1*; *Mavs* double mutants

Aberrant antiviral responses in the *Adar1* mutant are due to loss of RNA editing

Human *ADAR1* mutations causing AGS affect primarily the interferon-inducible isoform

We propose that inosine helps innate immunity to distinguish cellular from viral RNA

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The RNA-Editing Enzyme ADAR1 Controls Innate Immune Responses to RNA

Niamh M. Mannion,[1,7] Sam M. Greenwood,[1,7] Robert Young,[1] Sarah Cox,[1] James Brindle,[1] David Read,[1] Christoffer Nellåker,[2] Cornelia Vesely,[3] Chris P. Ponting,[2] Paul J. McLaughlin,[4] Michael F. Jantsch,[3] Julia Dorin,[1] Ian R. Adams,[1] A.D.J. Scadden,[5] Marie Öhman,[6] Liam P. Keegan,[1,*] and Mary A. O'Connell[1,6,*]
[1]MRC Human Genetics Unit, IGMM, University of Edinburgh, Crewe Road, Edinburgh EH4 2XU, UK
[2]MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, South Parks Road, Oxford OX1 3QX, UK
[3]Department of Chromosome Biology, Max F. Perutz Laboratories, University of Vienna, Dr. Bohr-Gasse 9/5, 1030 Vienna, Austria
[4]Institute of Structural and Molecular Biology, Michael Swann Building, School of Biological Sciences, Kings Buildings, West Mains Road, Edinburgh EH9 3JG, UK
[5]Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, UK
[6]Department of Molecular Biosciences, Wenner Gren Institute, Stockholm University, 106 91 Stockholm, Sweden
[7]Co-first author
*Correspondence: liam.keegan@igmm.ed.ac.uk (L.P.K.), mary.oconnell@igmm.ed.ac.uk (M.A.O.)
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SUMMARY

The ADAR RNA-editing enzymes deaminate adenosine bases to inosines in cellular RNAs. Aberrant interferon expression occurs in patients in whom *ADAR1* mutations cause Aicardi-Goutières syndrome (AGS) or dystonia arising from striatal neurodegeneration. *Adar1* mutant mouse embryos show aberrant interferon induction and die by embryonic day E12.5. We demonstrate that *Adar1* embryonic lethality is rescued to live birth in *Adar1*; *Mavs* double mutants in which the antiviral interferon induction response to cytoplasmic double-stranded RNA (dsRNA) is prevented. Aberrant immune responses in *Adar1* mutant mouse embryo fibroblasts are dramatically reduced by restoring the expression of editing-active cytoplasmic ADARs. We propose that inosine in cellular RNA inhibits antiviral inflammatory and interferon responses by altering RLR interactions. Transfecting dsRNA oligonucleotides containing inosine-uracil base pairs into *Adar1* mutant mouse embryo fibroblasts reduces the aberrant innate immune response. *ADAR1* mutations causing AGS affect the activity of the interferon-inducible cytoplasmic isoform more severely than the nuclear isoform.

INTRODUCTION

In vertebrates, viral double-stranded RNAs (dsRNAs) in the cytoplasm bind and activate RIG-I (retinoic acid-inducible gene I)-like cytoplasmic viral sensor proteins (RLRs) (for review, see Takeuchi and Akira, 2010). The known features that these sensors use to discriminate virus and pathogen RNAs from host cytoplasmic RNAs include the presence of dsRNA ends and 5′ triphosphates. RLRs translocate on dsRNA, and some RLRs may scan dsRNA to help distinguish between host and virus molecules. Cytoplasmic viral RNAs usually lack modifications, because most viruses do not encode modifying enzymes. It has been proposed that nucleic acid modifications of cellular RNAs help innate immune sensors to avoid aberrant activation by host nucleic acids (Gehrig et al., 2012; Karikó et al., 2005; Vitali and Scadden, 2010). Consistent with this idea, transfection of in-vitro-transcribed RNA into cultured cells generates an innate immune response. However, if the RNA is synthesized to contain naturally occurring modified bases, then the modified RNA does not cause innate immune induction (Karikó et al., 2005; Warren et al., 2010).

Suppression of responses of innate immune RNA sensors by modifications normally present in host RNAs could act as thresholding mechanisms to help prevent aberrant responses. Response thresholding mechanisms may be required because some cellular RNAs do contain RNA duplexes or have 5′ triphosphates. RNA duplexes in host RNAs are particularly hazardous; some Alu hairpins are still present in 3′ UTRs of mature mRNAs in the cytoplasm (Capshew et al., 2012). Transcription also occurs over most of the human genome, which inevitably generates further dsRNA that may reach the cytoplasm (Kapranov et al., 2007). The RIG-I and MDA5 (melanoma differentiation associated gene 5, IFIH1; interferon induced with helicase C domain 1) sensors are activated by binding RNA duplexes and signal through the MAVS (mitochondrial antiviral signaling) adaptor protein to activate NFκB, IRF3/7, and AP1. This activates transcription of genes encoding type I interferon (IFN) and proinflammatory cytokines. Secreted type I IFN binds to cell-surface type I IFN receptors to amplify and spread the antiviral response, inducing transcription of a large set of antiviral, IFN-stimulated genes (ISGs). Aberrant or chronic activation of IFN-stimulated defense processes is very damaging to the host.

Experiments in cultured cells do not test the overall importance of RNA modification for restraining innate immune responses in whole-model organisms or in human diseases.

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However, recent findings on human gene mutations causing autoimmune diseases are consistent with possible significant roles for RNA modifications in autoimmune diseases. For instance, mutations in the *ADAR1* gene encoding one RNA-editing *a*denosine *dea*minase acting on *R*NA cause Aicardi-Goutières syndrome (AGS) (Rice et al., 2012). AGS is a fatal childhood encephalopathy with aberrant IFN expression and symptoms resembling those caused by congenital virus infection. Similar rare mutations in *ADAR1* also cause dystonia due to bilateral striatal neurodegeneration associated with IFN overproduction (Livingston et al., 2014). Over 100 mutations in *ADAR1* have been identified in East-Asian patients with dyschromatosis symmetrica hereditaria (DSH), a mild genodermatosis with mostly unknown IFN status, in which the dominant phenotype appears to be often due to *ADAR1* haploinsufficiency (Liu et al., 2006).

ADARs catalyze the deamination of adenosine to inosine, which is the most common base modification known to occur in mammalian RNA (Gerber and Keller, 2001). Inosine is readily detected in transcriptome sequence data because inosine prefers to base pair with cytosine, leading to replacement of genome-encoded adenosine (A) by guanosine (G) at edited positions in cDNA sequences. ADARs edit particular adenosine residues at specific positions in short RNA duplexes in protein-coding transcripts, and they also edit numerous adenosines promiscuously in longer dsRNAs (for review, see Heale and O'Connell, 2009). Site-specific RNA-editing events in transcript open reading frames that generate new isoforms of encoded proteins represent the best-understood mechanism of ADAR action. Vertebrates have two enzymatically active ADAR proteins: ADAR1 (*ADAR*) and ADAR2 (*ADARB1*). Editing of the critical Q/R site in the transcript encoding the key AMPA receptor subunit is performed by ADAR2. Mutant *Adar2* mice die from seizures within 3 weeks of birth, and seizures and death are prevented by knocking in an editing-equivalent A to G mutation in the AMPA receptor subunit gene (Higuchi et al., 2000).

Adar1 mutant mice die by embryonic day E12.5 with massive overproduction of IFN, loss of embryonic liver hematopoietic cells, liver disintegration, and widespread apoptosis (Hartner et al., 2004; Wang et al., 2004). Cultured *Adar1* mutant embryonic fibroblasts are also highly sensitive to stress-induced apoptosis (Hartner et al., 2004; Wang et al., 2004). For many years, research has focused on identifying a key ADAR1 target transcript analogous to the AMPA receptor transcript. However, among the approximately 60 known editing events that recode open reading frames, many of the key events are catalyzed primarily by ADAR2, and none appears likely to account for the *Adar1* mutant embryonic lethal phenotype. However, only 0.4% of human A-to-I editing occurs within protein-coding sequences (Peng et al., 2012); the vast majority of known A-to-I-editing sites, now estimated at over 100 million in the human genome (Bazak et al., 2013), have been found in RNA duplex-forming pairs of Alu elements embedded in inverted orientations near each other in introns and 3′ UTR regions of transcripts.

Editing of cellular dsRNA leads to formation of I-U wobble base pairs that cause bending and alter the properties of the dsRNA helix; multiple sequential I-U base pairs and high levels of promiscuous editing destabilize dsRNA. Editing of endogenous RNA duplexes may lower the risk that they aberrantly induce innate immune responses. The constitutive ADARp110 isoform is a shuttling protein that accumulates mainly in the nucleus and edits dsRNA before nuclear export (Desterro et al., 2003). The N-terminally extended ADARp150 isoform, expressed from a late IFN-inducible promoter, is predominantly cytoplasmic and has been shown to edit viral RNAs (Samuel, 2011). Transfected dsRNA oligonucleotides containing inosine-uracil (I:U) base pairs have been shown to bind to RLRs competitively with poly I:C and to suppress activation of innate immune responses (Vitali and Scadden, 2010).

We reveal that loss of ADAR1 RNA-editing activity and the resulting loss of inosine bases in RNA are critical in producing aberrant RLR-mediated innate immune responses in *Adar1* mutant mice and cultured mouse cells. We characterize the immune response-blocking actions of human ADAR1 protein in *Adar1* mutant mouse cells and show that most AGS-associated ADAR1 mutant proteins have impaired RNA-editing activity.

RESULTS

IFN Receptor Mutation Partially Rescues Mouse *Adar1* Mutant Embryonic Lethality, whereas *Mavs* Mutation Rescues to Live Births

We wished to investigate whether aberrant immune responses are critical to the *Adar1* mutant embryonic lethal phenotype in mice. Preventing type I IFN signaling is sufficient to fully rescue embryonic lethality in embryos mutant for *DNaseII* (Yoshida et al., 2005) that also die with type I IFN overproduction. We constructed mouse strains that generate *Adar1*; *Ifnar1* (IFN-α and -β receptor 1) double-mutant embryos in crosses. No live *Adar1*$^{-/-}$; *Ifnar1*$^{-/-}$ progeny were obtained (Table S1). Staged embryo collections from crosses of *Adar1*$^{+/-}$; *Ifnar1*$^{-/-}$ parents suggest that the embryonic lifespan of the double homozygous *Adar1*$^{-/-}$; *Ifnar1*$^{-/-}$ embryos was extended due to *Ifnar1*$^{-/-}$ (Table 1; Figure 1); the mixed C57Bl6/129 strain background in these crosses had little effect (Table S1). Responses to secreted type I IFN are not the main reason for *Adar1* mutant embryonic lethality.

Although embryonic death is delayed, histological analysis of the *Adar1*; *Ifnar1* embryos revealed that the rescued embryos die with defects similar to those seen in the *Adar1* mutant (Figure S1A). Rescue is incomplete and variable at E14.5–E15.5 (Figures 1A–1F), and some embryos still have major defects in liver structure with apoptotic nuclei both in hepatocytes and hematopoietic cells (Figures 1E–1H). Liver, heart, and lungs are underdeveloped in *Adar1*; *Ifnar1* embryos compared to wild-type; there are fewer densely staining hematopoietic cells in the *Adar1*; *Ifnar1* liver, and there is an apparent lack of peripheral blood in or around any of the organs (Figure 1). In the E15.5 *Adar1*; *Ifnar1* liver, 58% of the erythrocytes are still nucleated, versus ∼5% in the wild-type (Figure S1B). A highly similar phenotype was observed in *Adar1*$^{-/-}$; *Stat1*$^{-/-}$ embryos, with hematopoietic defects and subsequent lethality occurring around E15.5 (Figure S1C; Table S1). *Stat1* is a key mediator of systemic IFN responses (Stark and Darnell, 2012). Embryonic lethality occurs earlier in *Adar1* mutant than in the *DNaseII* mutant and does not depend on the amplifying effect of high type 1 IFN secretion.

Table 1. Survival of Mouse Embryos with Different *Adar1* Genotypes from Crosses between *Adar1*$^{+/-}$; *Ifnar1*$^{-/-}$ (C57Bl6N/129) or *Adar1*$^{+/-}$; *Mavs*$^{-/-}$ (C57Bl6N/J) Parents

Day	Litters	Embryos	*Adar1*$^{+/+}$	*Adar1*$^{+/-}$	*Adar1*$^{-/-}$	Exp. *Adar1*$^{-/-}$
Parents crossed; *Adar1*$^{+/-}$; *Ifnar1*$^{-/-}$						
E 11.5	2	14	4	7	3	3.50
E 12.5	2	13	6	7	0	3.25
E 13.5	4	22	4	14	4	5.50
E 14.5	4	28	8	15	5	7.00
E 15.5	5	33	16	16	1	8.25
E 16.5	2	9	0	6	3	2.25
E 17.5	2	10	5	5	0	2.50
Total		129	43	70	16	
Parents crossed; *Adar1*$^{+/-}$; *Mavs*$^{-/-}$						
E 11.5	7	59	17	32	10	14.75
E 12.5	2	15	4	7	4	3.75
E 13.5	2	19	5	10	4	4.75
E 14.5	2	11	3	7	1	2.75
E 15.5	4	29	8	15	6	7.25
P 0.5	7	60	17	35	8	15.00
Total		193	54	106	33	

Because *Adar1*; *Ifnar1* double-mutant embryos do survive for 3–4 additional days, this suggested that rescue of *Adar1* mutant embryonic lethality might be achieved with a mutation giving a more potent, cell autonomous block to aberrant immune antiviral activation, IFN production, and apoptosis. If cytoplasmic dsRNA in the *Adar1* mutant triggers an aberrant antiviral response by activating RLRs, then the key adaptor protein in that signaling pathway is the mitochondrial antiviral signaling protein MAVS. To investigate the effect of blocking signaling from RLRs, we generated strains that produce *Adar1*$^{-/-}$; *Mavs*$^{-/-}$ double-mutant embryos in crosses. The *Adar1*; *Mavs* double-mutant embryos have a significantly extended survival up to live birth of pups (Table 1; Figure 2A). Histological sections through a newborn *Adar1*; *Mavs* pup show apparently normal liver, heart, and other internal organs (Figure 2A). The *Adar1*; *Mavs* double-mutant pups are able to feed, but they die within a day of birth. Preliminary data suggest that the newborn pups still show heightened IFN and interleukin 1 (IL-1) expression in blood (data not shown). This rescue shows that *Adar1* mutant embryonic lethality is largely due to aberrant RLR/MAVS pathway signaling triggering apoptosis.

To investigate innate immune responses in more detail, six primary mouse embryonic fibroblast (MEF) cultures were established representing all possible *Adar1* genotypes in *Mavs* mutant or wild-type genetic backgrounds. Constitutive and polyriboinosinic:polyribocytidylic acid (poly I:C)-induced levels of IFN-α and interleukin-6 (IL-6) proteins were measured in early-passage MEF culture supernatants by ELISA. *Adar1*$^{-/-}$; *Mavs*$^{+/+}$ MEFs show a detectably elevated basal level of IFN-α and respond to poly I:C by producing higher levels of both IFN-α and IL-6 than the wild-type (*Adar1*$^{+/+}$; *Mavs*$^{+/+}$) MEFs. Surprisingly, heterozygous *Adar1*$^{-/+}$ MEFs behave somewhat like homozygous *Adar1*$^{-/-}$ mutant MEFs and respond vigorously to poly I:C treatment with heightened expression of both IFN-α and IL-6. This suggests that prevention of aberrant immune responses in *Adar1*$^{-/+}$ MEFs is very sensitive to levels of ADAR1, consistent with evidence of *ADAR1* haploinsufficiency in DSH patients (Liu et al., 2006). Confirming that *Mavs* mutation blocks the aberrant innate immune responses caused by the *Adar1* mutation, aberrant expression of type I IFN and proinflammatory cytokines observed in *Adar1*$^{-/-}$;*Mavs*$^{+/+}$ MEFs is prevented in the *Adar1*$^{-/-}$; *Mavs*$^{-/-}$ double-mutant MEFs; these MEFs also do not induce type I IFN or IL-6 in response to poly I:C treatment (Figure 2B).

Transcriptional Alterations in *Adar1* Mutant Embryos Are Primarily Consequences of Aberrant Immune Induction

To investigate whether there is an altered transcriptional profile in *Adar1* mutant embryos quantitative RT-PCR (qRT-PCR) analyses were performed to determine the expression levels of 12 interferon-stimulated gene (ISG), transcripts in total RNA from E11.5 whole embryos. Some ISG transcripts are highly elevated in *Adar1* mutant whole embryos compared to wild-type embryos and levels return to normal in *Adar1*; *Mavs* and *Adar1*; *Ifnar* embryos (Figure 3A), though the *Adar1*; *Ifnar1* double-mutant embryo does not normalize ISG transcript levels as fully as *Adar1*; *Mavs*. The levels of these ISG transcripts in the *Mavs* and *Ifnar1* mutant embryos are also somewhat different from wild-type levels (Figure S2), and therefore the relevant background has been subtracted from the double-mutant levels (Figure 3A). The qRT-PCR data confirm the ELISA data on cultured fibroblasts showing that the abnormal IFN responses in *Adar1* mutant embryos are mediated by aberrant immune signaling through the MAVS adaptor protein.

To characterize alterations in gene expression across the whole transcriptome in *Adar1* mutant *Adar1*; *Mavs* double-mutant embryos, we sequenced ribosomal RNA-depleted total RNA from whole E11.5 embryos. Overall, more transcripts decrease in whole *Adar1* embryos than increase (Figure 3B); this may be related to losses of hematopoietic cells or to more widespread defects consistent with apoptosis observed in *Adar1* mutant embryos. We identified a set of 61 protein-coding transcripts that are upregulated at least 3-fold in *Adar1* mutant embryos and restored to near-wild-type levels in *Adar1*; *Mavs* embryos (Table S2). Gene Ontology term analysis on the upregulated transcripts confirms enrichment of immune and antiviral response transcripts (Table S2). To confirm the increased expression of immune gene transcripts in the *Adar1* mutant, qRT-PCR expression analyses were performed with total RNA from E11.5 whole embryos. Changes in proinflammatory cytokine transcript expression in *Adar1*; *Ifnar1* and *Adar1*; *Mavs* embryos were calculated relative those in *Ifnar1* and *Mavs* mutants, which were again slightly different from wild-type levels (Figure S2). Consistent with the sequencing data, levels of certain ISGs and proinflammatory cytokines were highly increased in the *Adar1* mutant compared with wild-type (Figures 3A and 3B).

Altered expression levels of immune gene transcripts in the *Adar1* mutant embryo are restored close to wild-type levels in

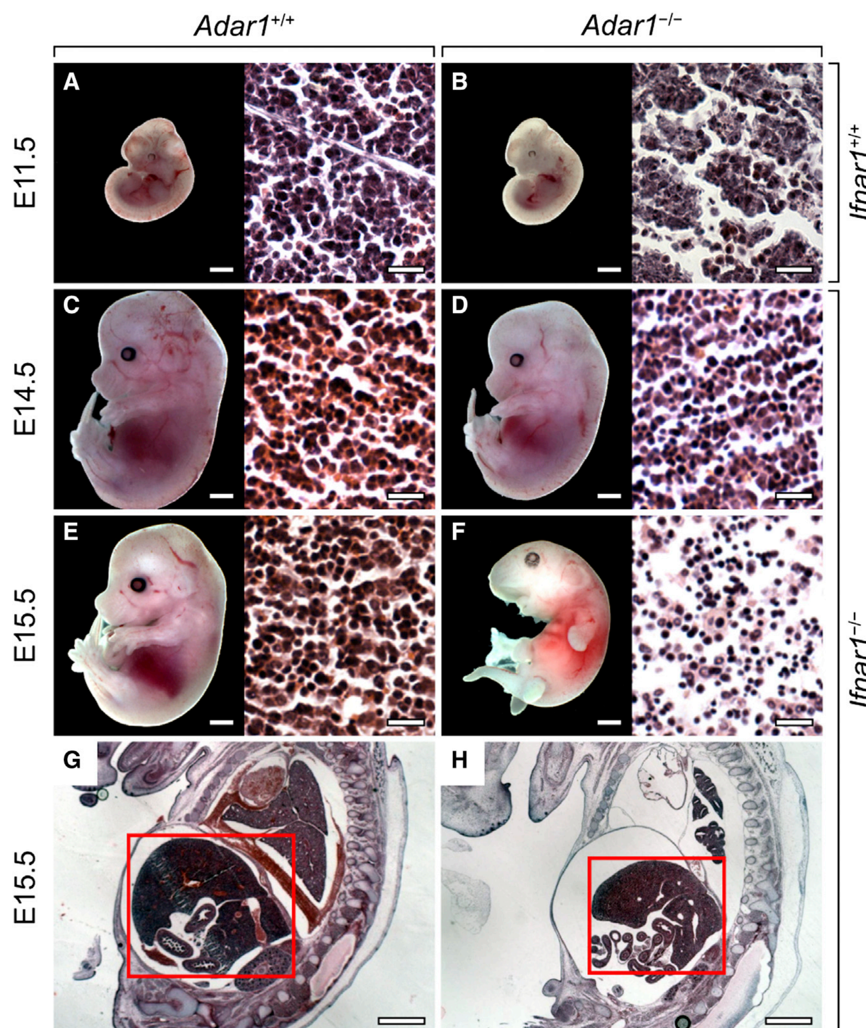

Figure 1. Partial Rescue of *Adar1* Mutant Embryo Viability and Liver Integrity in *Adar1*; *Ifnar1* Double Mutant

(A and B) Whole embryos and corresponding liver sections of wild-type (A) and *Adar1* mutant (B) mice at E11.5.

(C–F) Whole embryos and corresponding liver sections of *Adar1*; *Ifnar1* (C and E) and *Adar1*; *Ifnar1* (D and F) mice at E14.5 (C and D) and E15.5 (E and F).

(G and H) Sectioned whole embryos of $Adar1^{+/+}$; $Ifnar1^{-/-}$ (G) and $Adar1^{-/-}$; $Ifnar1^{-/-}$ (H) mice at E15.5. Fetal livers outlined by red boxes.

Scale bars, liver sections (A–F), 25 μm; all others, 1 mm.

both *Adar1*; *Ifnar1* and *Adar1*; *Mavs* double-mutant embryos. The increase in the expression of the transcription factor *Stat1* is very striking in the *Adar1* embryo at E11.5 and the *Stat1* transcript is reduced in both *Adar1*; *Ifnar1* and *Adar1*; *Mavs* double-mutant embryos. STAT1 protein is critical to the cellular response to extracellular IFN (for review, see Stark and Darnell, 2012); however, consistent with the similarly limited effect of the *Ifnar1* mutation, generating an *Adar1*; *Stat1* double mutant also does not rescue *Adar1* mutant embryonic lethality fully (Figure S1C). The stronger rescue of embryonic lethality by *Mavs* mutation may not be due mainly to stronger effects on transcription, but rather the *Mavs* mutation may also block apoptosis or other nontranscriptional effects of aberrant RLR signaling. Together both the qRT-PCR and ELISA data confirm that the abnormal responses in *Adar1* mutant embryos are mediated by immune signaling through MAVS.

To examine transcription in more detail in embryonic hematopoietic cells, we sequenced ribosomal RNA-depleted total RNA from E15.5 embryonic livers of *Adar1*; *Mavs* and *Mavs* sibling embryos; liver is the main site of hematopoiesis at this stage. *Adar1* mutant embryos die by E12.5 when embryonic liver is still small. *Adar1*; *Mavs* E15.5 fetal livers appear normal and comparisons of RNA sequencing (RNA-seq) data from *Adar1*; *Mavs* and *Mavs* livers could not identify any protein-coding transcripts expressed at significantly differing levels. The lack of aberrant transcription in *Adar1*; *Mavs* livers shows that all observed alterations in levels of protein-coding transcripts in the *Adar1* mutant are effects of aberrant RLR signaling and interferon production. Transcriptional alterations are not caused directly by loss of ADAR1 protein per se.

It has been proposed that RNA editing could increase turnover of edited dsRNA due to cleavage by an inosine-specific ribonuclease, but the physiological effectiveness of this turnover process is not known (Scadden, 2005). If increased levels of repetitive transcripts with dsRNA-forming potential do arise in the *Adar1* mutant, these might cause the aberrant antiviral response. If repetitive transcripts increase as a direct result of the absence of *Adar1*, the aberrant repetitive transcript levels should be detected also in the *Adar1*; *Mavs* double mutant. To assess expression of transcripts likely to form dsRNA, a detailed analysis of repetitive sequence expression in *Adar1*; *Mavs* and *Mavs* E15.5 liver total RNA-seq data was performed (Figure 4). Increased expression of individual members of ERV and IAP subfamilies was observed (Table S3), and qRT-PCR analyses confirmed increased expression of MMERVK10C transcripts (Figure 4E). Overall, however, changes in repetitive transcripts parallel those seen for protein-coding transcript; i.e., levels of most repetitive elements did not change between *Adar1*; *Mavs* and *Mavs*. This suggests that a general dramatic increase in levels of repetitive transcripts due to slowed turnover is not the initiating event in the aberrant immune response in *Adar1* mutants. Changes in some individual repetitive transcripts could contribute to initiating aberrant immune responses in the *Adar1* mutant, but the reduction in inosine levels within cellular dsRNA is perhaps more critical.

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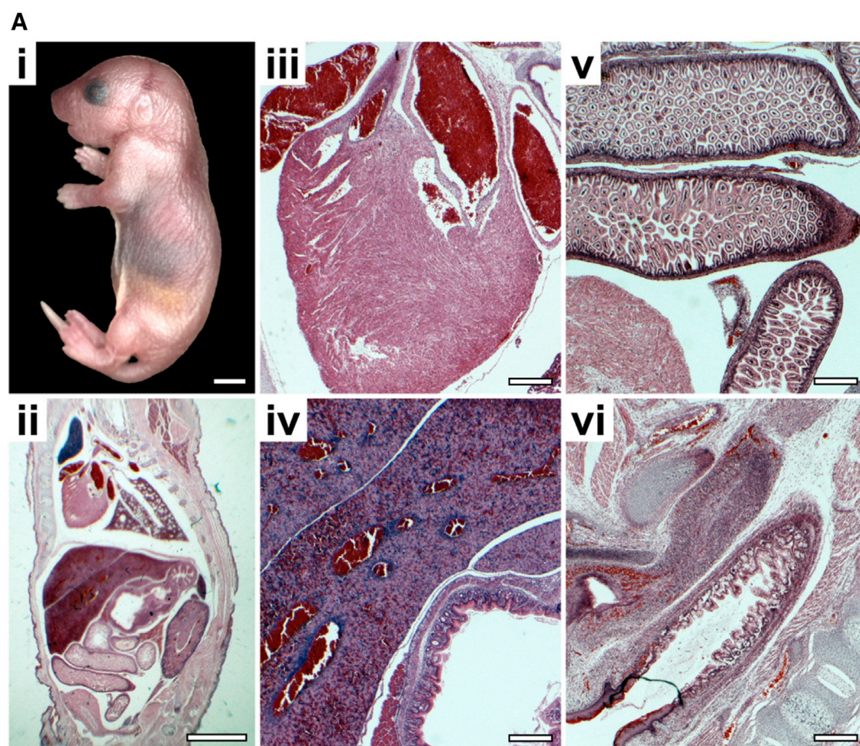


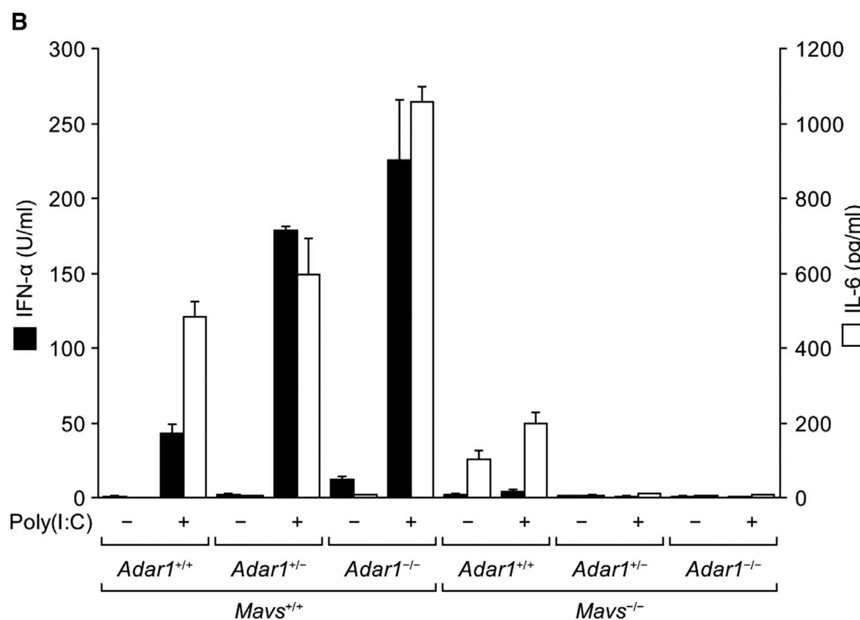


Figure 2. Rescue of *Adar1* Mutant Phenotypes in *Adar1*; *Mavs* Double-Mutant Mice and MEF Cultures

(A) Gross visceral anatomy of *Adar1*$^{-/-}$; *Mavs*$^{-/-}$ newborn mice. (i) Appearance of pups at P0.5. (ii–vi) Sections showing general visceral anatomy (ii) with further detail of heart (iii), liver (iv), intestines (v), and rectum (vi). Scale bars in (i) and (ii), 3 mm and (iii)–(vi), 400 μm.

(B) ELISA showing mean levels of IFN-α and IL-6 in cell-culture supernatants of *Adar1*$^{+/+}$, *Adar1*$^{+/-}$, and *Adar1*$^{-/-}$ MEFs with *Mavs*$^{+/+}$ or *Mavs*$^{-/-}$ backgrounds following transfection with poly(I:C) (1 μg/ml; +) or water (−). The units on the y axis are expressed per 10,000 cells. Error bars, SD.

Restoring Expression of Editing-Competent ADARs Prevents Aberrant Innate Immune Responses in *Adar1* Mutant Mouse Fibroblasts

To determine whether the aberrant antiviral response in *Adar1* mutant cells is due to reduced inosine modification in intracellular RNAs, we established MEF cell cultures from *Adar1*; *p53* double-mutant embryos. *Adar1* MEFs could not be cultured long term due to cell death (Figure S3), and *Adar1*; *Mavs* double-mutant MEFs are not useful because the aberrant innate immune response is blocked. When compared with *p53* mutant MEFs, elevated expression of ISGs is observed in *Adar1*; *p53* MEFs when cultures are stressed by nutrient starvation (Figure 5A); without stress, ISG expression is very low in *Adar1*; *p53* MEFs. We chose the *p53* mutant background because this prevents cell death associated with a range or other mutations (Dittmer et al., 1993). The reduction in aberrant ISG expression in *Adar1*; *p53* versus *Adar1* MEFs is consistent with the idea that preventing cell death is also an important feature of the *Mavs* mutant rescue.

To test for rescue of *Adar1* mutant phenotypes by ADAR protein variants, we next established stable *Adar1*; *p53* MEF lines expressing wild-type or mutant human ADAR1 proteins from stably integrated PiggyBac constructs that we previously characterized (Heale et al., 2009). The *Adar1*; *p53* PiggyBac MEFs were starved for 72 hr to stress the cells, total RNA was isolated and qRT-PCR was performed to measure ISG transcript levels. Expression levels of ISG transcripts were normal in *p53* mutant cells and highly elevated in *Adar1*; *p53* cells (Figure 5A). When either the predominantly nuclear ADAR1 p110 isoform or the IFN-inducible mainly cytoplasmic ADAR1p150 isoform were expressed, ISG transcripts were significantly reduced (Figure 5A). Expressing the ADAR1p150 (E912A) mutant that inactivates the deaminase catalytic site gave the least effective reduction in ISG levels. These data indicate that robust suppression of ISG transcription requires a catalytically active ADAR capable of deaminating adenosine to inosine into RNA.

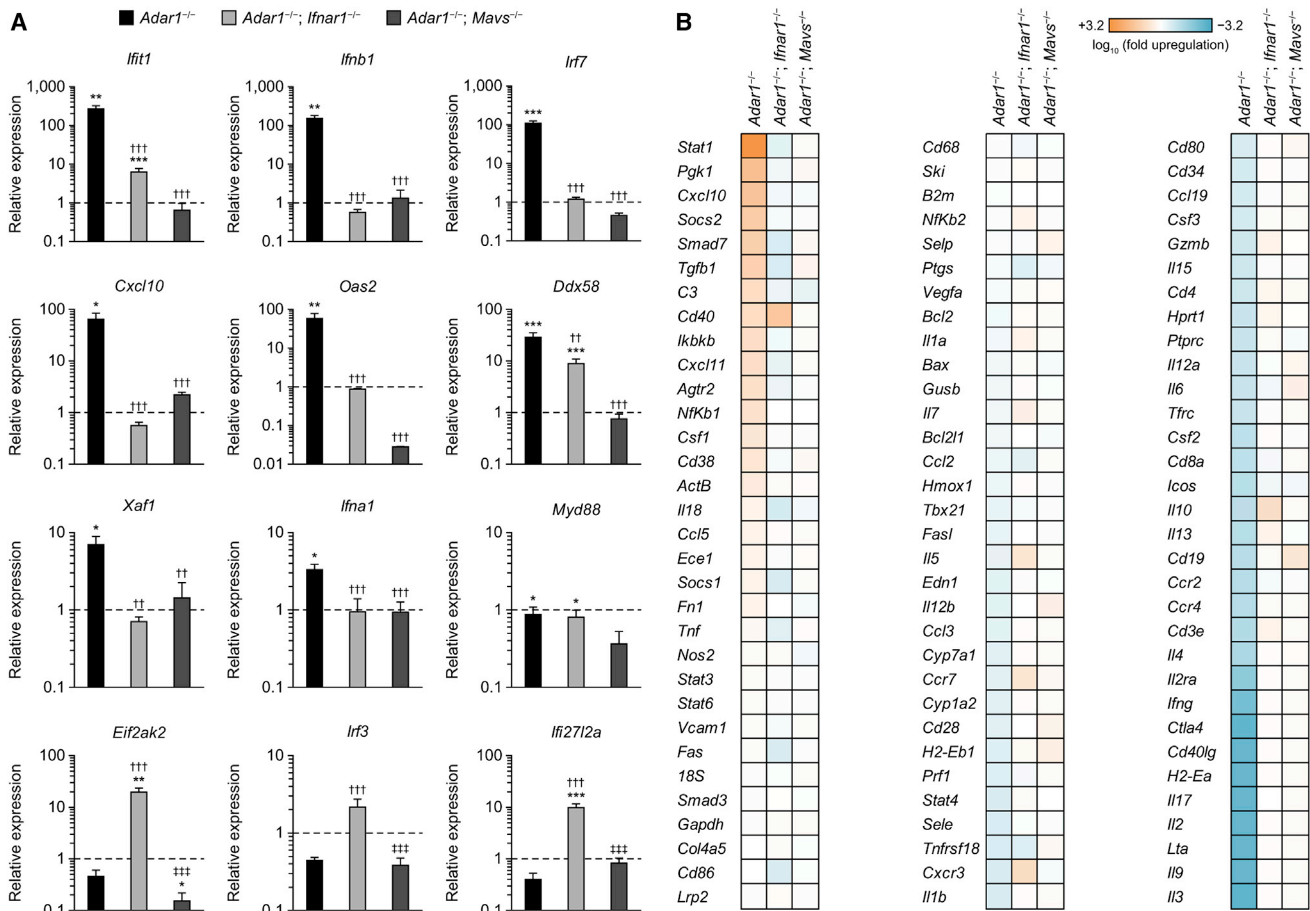


Figure 3. Rescue of *Adar1* Mutant Embryo Aberrant Proinflammatory Cytokine Expression in *Adar1*; *Ifnar1* and *Adar1*; *Mavs* Double-Mutant Embryos

(A) Expression levels for an array of 12 ISGs measured in E11.5 whole embryos. For each gene, *Adar1*$^{-/-}$, *Adar1*$^{-/-}$; *Ifnar1*$^{-/-}$, and *Adar1*$^{-/-}$; *Mavs* values are expressed relative to wild-type, *Ifnar1*, or *Mavs* background levels, respectively (one on y axis, dashed lines). Error bars, SEM. *p ≤ 0.05; **p < 0.01; ***p < 0.001 versus wild-type; ††p < 0.01; †††p < 0.001 versus *Adar1*; ‡‡‡p < 0.001 versus *Adar1*$^{-/-}$; *Ifnar1*$^{-/-}$.

(B) Heatmap showing expression levels for an array of 96 proinflammatory cytokine and control transcripts measured in E11.5 whole embryos. For each gene, *Adar1*$^{-/-}$, *Adar1*$^{-/-}$; *Ifnar1*$^{-/-}$, and *Adar1*$^{-/-}$; *Mavs*$^{-/-}$ values are expressed relative to wild-type, *Ifnar1*$^{-/-}$, or *Mavs*$^{-/-}$ background levels, respectively, which are all zero following $\log_{10}$-transformation and hence are not shown (white in color).

Expressing human ADAR2ΔN, a nuclear localization mutant of ADAR2 that aberrantly accumulates in the cytoplasm (Wong et al., 2003), in *Adar1*; *p53* MEFs also significantly reduced expression of the ISGs (Figure 5A). This suggests that restoring RNA-editing activity in the cytoplasm is particularly important. MEF cells already express endogenous, active, ADAR2 in the nucleus, and site-specific editing of protein-coding transcripts usually involves transient exon-intron RNA duplexes formed before splicing. ADAR2 proteins have different specific site preferences from ADAR1 proteins (Keegan et al., 2011). The ADAR2ΔN is more likely to rescue nonspecific, promiscuous editing events lost in the *Adar1* mutant than it is to edit some transcript containing a highly ADAR1-specific site. The sufficiency of either catalytically active ADAR for strong rescue is consistent with the idea that loss of promiscuous editing in intracellular dsRNA is critical to the *Adar1* mutant phenotype.

Transfecting dsRNA Containing I-U Base Pairs Prevents Aberrant Antiviral Responses in *Adar1* Mutant Mouse Fibroblasts

If cellular dsRNA lacking inosine in *Adar1* mutant cells is sufficient to induce an aberrant innate immune response, then isolating RNA from *Adar1* mutant cells and transfecting it into wild-type cells might replicate this effect. Therefore, we extracted total RNA from livers of *Adar1*; *Mavs* E15.5 embryos and transfected this into RIG-I reporter cells but could not detect any difference between the responses induced by *Adar1*; *Mavs* and wild-type RNA samples. Because the majority of a total RNA sample is rRNA or tRNAs, this experiment may not mimic in vivo conditions. We decided to perform instead the converse experiment, which was to determine if inosine-containing RNAs interfere with immune induction by transfected RNA.

To elucidate if transfecting dsRNA oligonucleotides containing inosine-uracil base pairs suppress aberrant ISG expression in

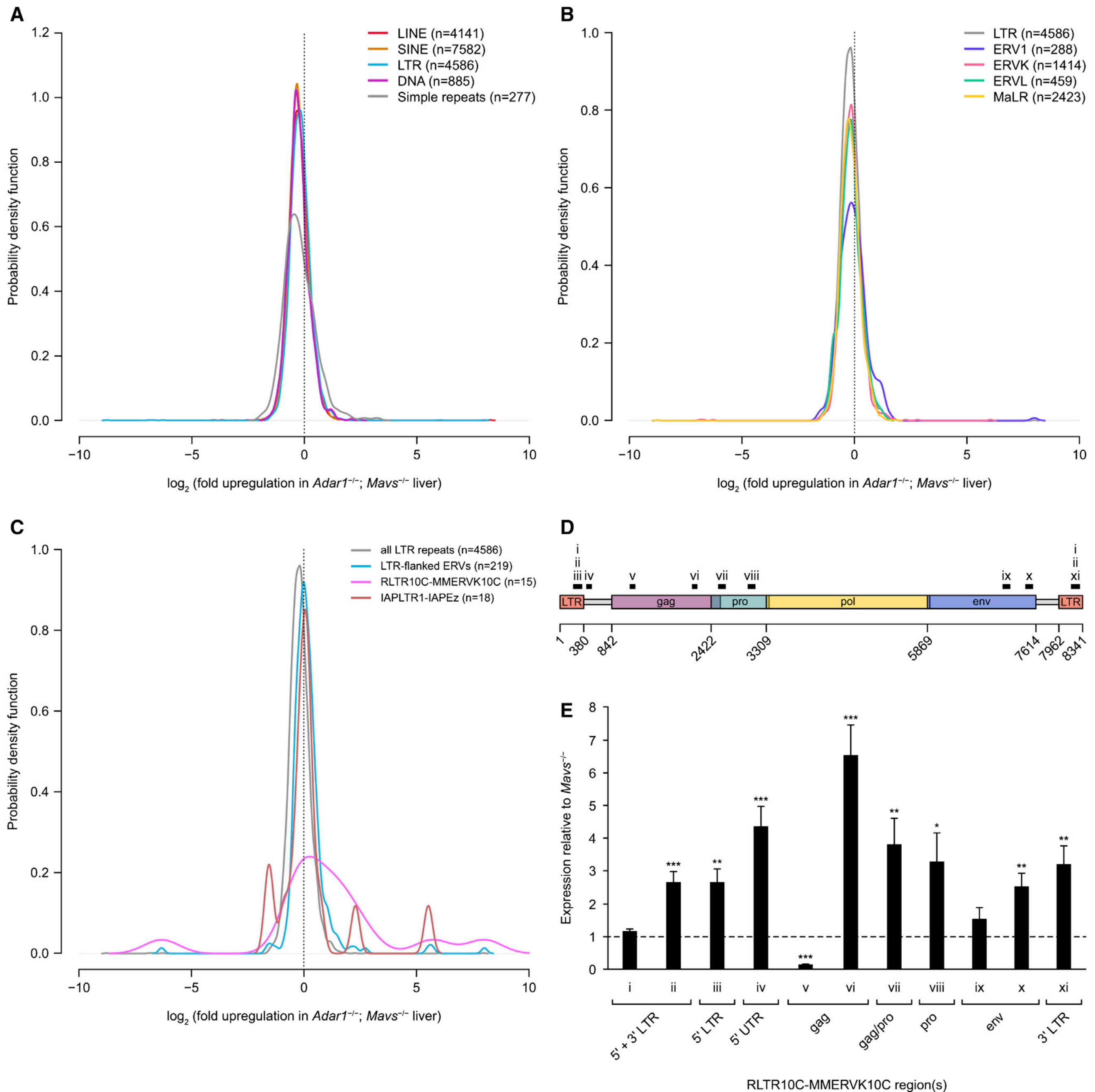


Figure 4. Repetitive Element Expression in *Adar1*; *Mavs* Embryonic Mouse Liver

(A–C) Expression profiles of repetitive element classes (A), retrotransposon subfamilies (B), and specific LTR retrotransposon populations (C) in *Adar1*$^{-/-}$; *Mavs*$^{-/-}$ E15.5 liver total RNA relative to *Mavs*$^{-/-}$ (zero on x axis).

(D) Schematic of the prototypical mouse RLTR10C-flanked MMERVK10C retrotransposon (generated using Jurka et al., 2005). Positions and lengths of the *gag*, *pro*, *pol*, and *env* genes are shown. Black bars indicate qRT-PCR products (i–xi) generated using specific primer pairs (Table S4).

(E) Expression levels of RLTR10C-flanked MMERVK10C retrotransposon regions shown in (D) in *Adar1*$^{-/-}$; *Mavs* $^{-/-}$E15.5 livers relative to *Mavs*$^{-/-}$ (one on y axis, dashed line).

Error bars, SEM. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ versus *Mavs* for each region.

Adar1 mutant phenotype, we established a test system using *Adar1*; *p53* MEFs. To partially induce innate immune responses in *Adar1*; *p53* MEFs, we transfected these cells with levels of an in-vitro-transcribed *Fluc* mRNA that has been shown previously to induce a modest innate immune response in HeLa cells (Vitali and Scadden, 2010). Transfecting similar concentrations

of *Fluc* RNA into the hypersensitive *Adar1*; *p53* MEF cells caused a dramatic increase in expression of ISG transcripts (Figure 5B), as in the case of serum starvation (Figure 5A). This strong response of *Adar1*; *p53* MEFs to transfected RNA is likely to reflect the aberrant immune induction due to the *Adar1* mutation.

Transfecting dsRNA oligos alone into *Adar1*; *p53* MEFs has very little effect on immune induction at the concentrations used (Figure 5C). dsRNA oligonucleotide (C-dsRNA) or IU-dsRNA that contained four inosine-uracil base pairs were then cotransfected with 500 ng *Fluc* RNA to look for specific inhibitory effects of IU-dsRNA oligonucleotides on ISG transcript induction (Figure 5C). IU-dsRNAs further reduce ISG transcript expression below reductions caused by control C-dsRNAs (Figure 5D). These reductions in ISG transcript levels were maintained for 24 hr with the IU-dsRNA, whereas the C-dsRNA was unable to reduce the ISG response at this time point (data not shown). The background inhibitory effect of the control C-dsRNA oligonucleotide in these experiments could arise if the 20-mer dsRNA oligonucleotides bind only one RLR molecule each, thus impeding oligomerization of RLR CARD domains (Peisley et al., 2014). It is clear that transfection of I-U dsRNA specifically inhibits aberrant immune induction further. Both this specific effect of I-U base pairs and the general suppressive effect of 20-mer dsRNA oligonucleotides indicate that aberrant RNA complexes formed by RLRs are central to the *Adar1* mutant phenotype.

Mutations in Human *ADAR1* Associated with AGS Have More Severe Effects on the IFN-Inducible Isoform of ADAR1

Most of the mutations in ADAR1 occurring in patients with AGS change residues on the surface of the catalytic domain at the C terminus of the protein (Figure 6), close to where dsRNA is predicted to bind. Previously, we tested the effects of these AGS mutations on enzymatic activity in ADAR1p110 (Rice et al., 2012). Constructs expressing ADAR1p110 were transiently cotransfected into HEK293 cells with a construct expressing a known editing substrate for ADAR1 and editing activity was measured. Surprisingly, most of the AGS mutant proteins, with the exception of the *ADAR1 G1007R* mutation, still exhibited robust editing activity, though with statistically significant reductions.

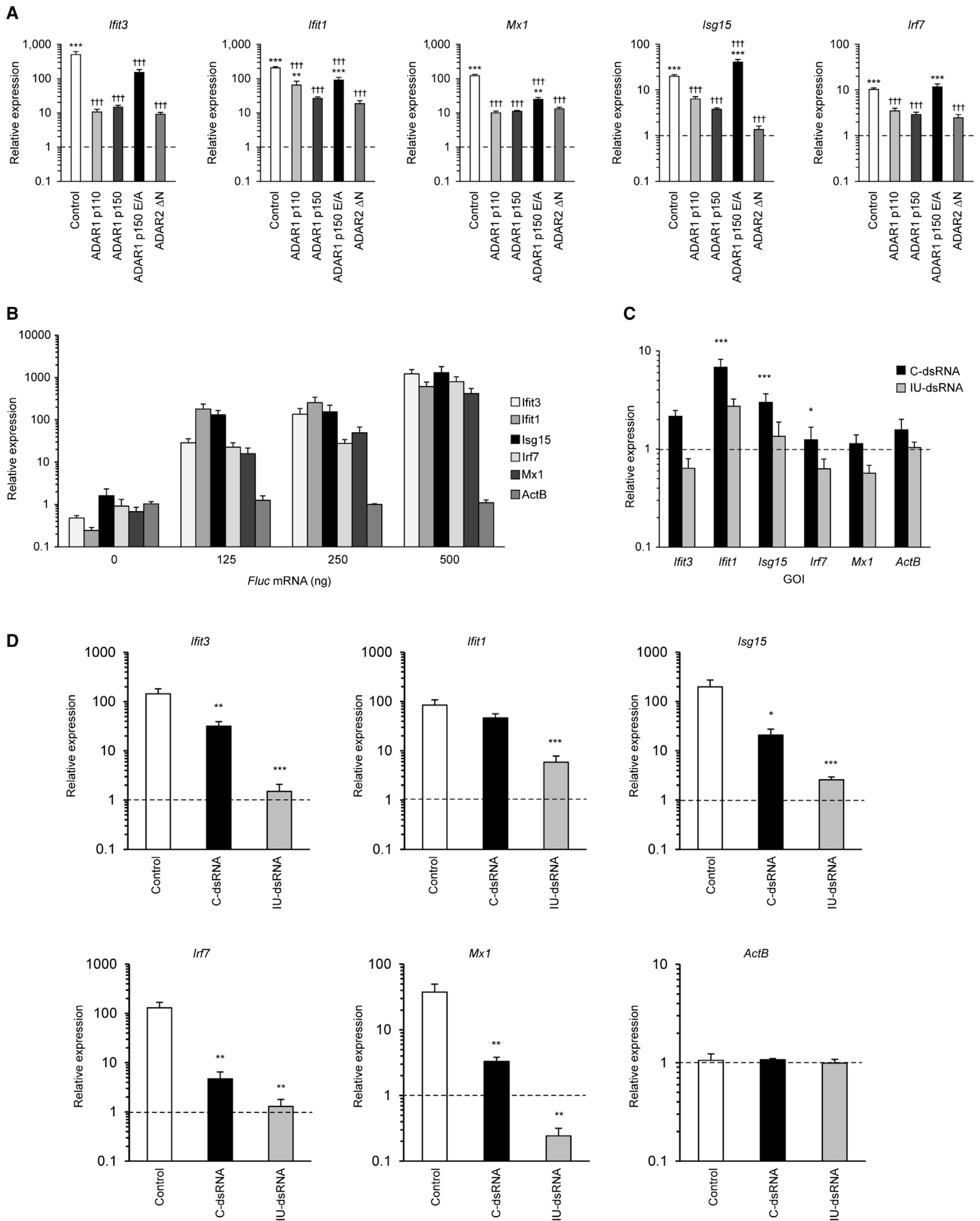
We now tested the effects of the same AGS mutations in ADAR1p150 in a similar manner (Figure 6A). Each of the AGS mutations causes a significant decrease in editing activity of ADAR1p150 that is greater than the effect of the same mutation in the ADAR1p110 isoform. This large difference between mutant effects in the two isoforms was not anticipated because the AGS mutations are predominantly in the C-terminal domains shared by the two isoforms. We provide a report of mutations in ADAR1 that have different effects on the two isoforms, revealing a significant separation of roles for the two isoforms. These findings also imply that the effects of AGS mutations in ADAR1 are mediated particularly through ADAR1p150 and primarily involve reductions in RNA-editing activity. Loss of the IFN-inducible cytoplasmic ADAR1 isoform may be important in AGS and dystonia patients. Clinical observations have suggested that symptoms may develop following an infection when type I IFN expression should have led to later expression of active ADAR1p150 (Livingston et al., 2014)

ADAR1 mutant proteins found in AGS patients were also tested for effects on RNA-editing activity in HEK293 cells when expressed in combinations matching those observed in the AGS patient cohort (Figure 6B). Evidence for interactions between different AGS mutations in the same ADAR1 protein and for interactions between different ADAR1 mutant proteins in heterozygotes was obtained. For instance, we found that the *ADAR1p150 P193A* mutant exhibits a significant decrease in editing activity (Figure 6A). The *P193A* mutation is expressed only in the N-terminally extended ADAR1p150 isoform and increases the effect of other AGS mutations in the same protein (Figure 6B). The crystal structure of the ADAR1 N-terminal Z-alpha domain complex with Z-RNA has been solved, and the *P193A* mutation is predicted to change a highly conserved amino acid involved in nucleic acid contact (Schade et al., 1999). This variant is enriched in the AGS cohort but is also present in 41 of 6,553 control patients in the Exome Variant Server database. An instance of strong interaction between different ADAR1 protein variants in AGS heterozygote patients is seen with the *ADAR1p110 G1007R* mutant that binds dsRNA but is catalytically inactive and which exerts a dominant-negative effect on editing by ADAR1p110 wild-type protein (Rice et al., 2012) (Figure 6B).

DISCUSSION

This study elucidates a key conserved role for ADARs in preventing aberrant IFN responses by cytoplasmic antiviral RLR innate immune RNA sensors. Mouse *Adar1* mutant embryos die by embryonic day E12.5 with aberrant IFN expression, failure of hematopoiesis, and degeneration of the embryonic liver (Hartner et al., 2004, 2009; Wang et al., 2004). We obtained partial rescues of *Adar1* mutant embryonic lethality by combination with *Ifnar1* or *Stat1* mutations and a much more substantial rescue to live birth by combination with a *Mavs* mutation. The *Mavs* rescue, in particular, indicates that the *Adar1* embryonic lethal mutant phenotype is largely due to an aberrant antiviral response. Some mutations affecting RLRs have phenotypes similar to *Adar1* mutants, for example, a mutation in the mouse *Ifih1* gene encoding MDA5 that causes constitutive IFN signaling (Funabiki et al., 2014), giving rise to a Lupus-like autoimmune disorder. Mutations in *Mavs* but not *Ifnar1* also rescued the aberrant immune response in those *Ifih1* mice. In addition, with striking similarity to *ADAR1*, mutations in human *IFIH1* also cause AGS (Rice et al., 2014). Our preliminary data indicate, however, that combining *Ifih1* with *Adar1* does not rescue the lethality of the *Adar1* mutant mouse embryo (data not shown), suggesting that MDA5 is not the sole mediator of the *Adar1* mutant phenotype.

Loss of the late IFN-inducible cytoplasmic ADAR1 p150 isoform, in particular, may be critical to the *Adar1* mutant phenotype. This isoform interacts in a complex way with viruses and may participate in the resolution phase of the IFN response by editing residual viral RNAs (Ward et al., 2011). ADARs have been reported as being "proviral" because ADARs facilitate replication of viruses in cultured cells (Samuel, 2011). However, proviral effects are unusual among proteins induced by IFN, and these experiments in cell cultures may be somewhat misleading. Studies on *Adar1* mutant mice lacking only the ADAR1 p150 isoform instead suggest an overall negative effect



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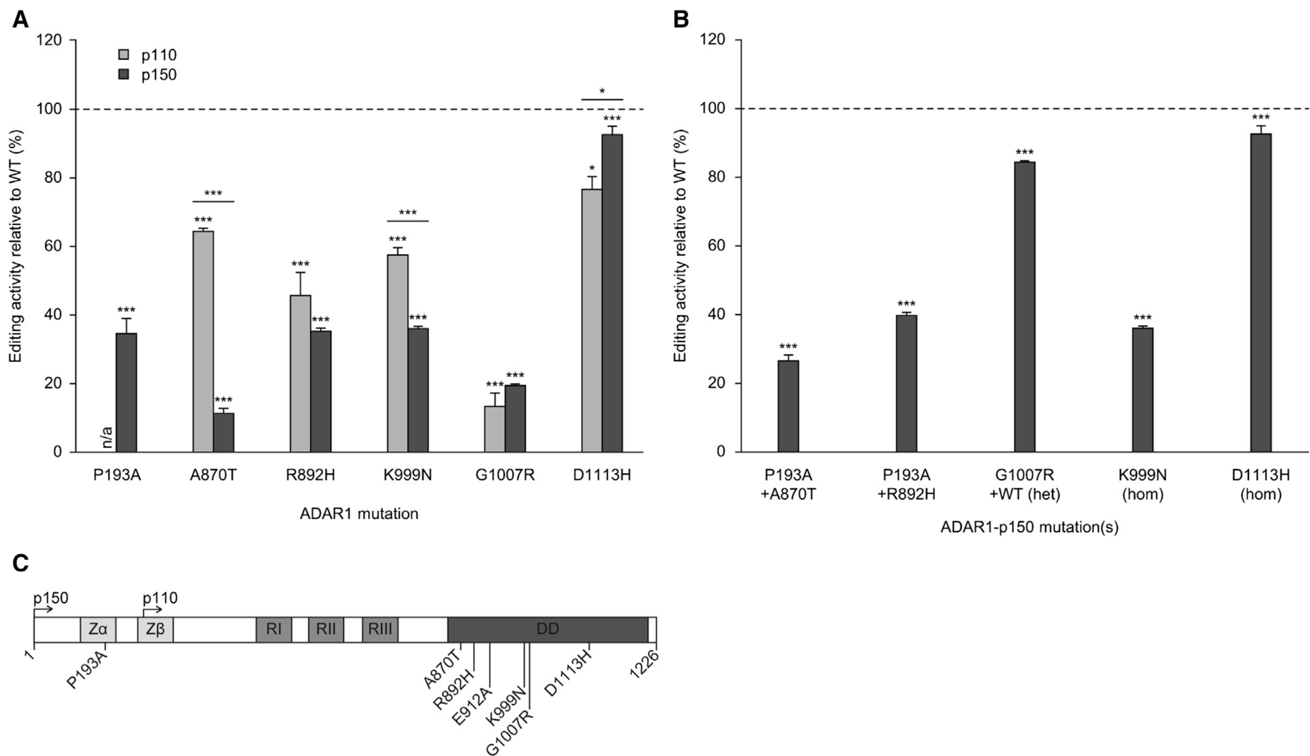

Figure 6. Mutations in ADAR1 that Cause Aicardi-Goutières Syndrome Affect RNA-Editing Activity

The first letter denotes the original amino acid and the second letter the mutation; the number is the position of the amino acid in the p150 isoform.

(A) Editing activity of each ADAR1 AGS mutant in either the p110 or p150 isoform expressed in HEK293T cells relative to wild-type p110 or p150, respectively (100% on y axis, dashed line). Error bars, SEM. $^{*}p \leq 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(B) Editing activity of the *ADAR1* mutant combinations found in an AGS patient cohort in the p150 isoform expressed in HEK293T cells relative to wild-type p150 (100% on y axis, dashed line). Error bars, SEM. $^{***}p < 0.001$ versus wild-type p150.

(C) A diagram of ADAR1 illustrating where the AGS mutations occur in the protein.

of ADAR1 p150 on measles virus propagation, illustrating the importance of whole-animal studies (Ward et al., 2011). Hepatitis C virus encodes a protease that cleaves MAVS, and it would be interesting to determine whether this virus or protease rescue aspects of *Adar1* mutant phenotypes.

We show that signaling from antiviral innate immune sensors through MAVS is inhibited by dsRNA oligonucleotides containing inosine-uracil base pairs. Transfection of I-U dsRNA corresponding to an ADAR1-edited section of an artificial dsRNA substrate into *Adar1* mutant MEFs suppresses the aberrant antiviral response. The data suggest that the aberrant antiviral response in the *Adar1* mutant results from loss of inosine in cytoplasmic dsRNA that normally inhibits antiviral sensor activation. Other examples of how changes in modification states of RNAs determine innate immune responses come from viruses encoding ribose 2′-*O*-methylation enzymes that methylate viral mRNA cap structures to match host mRNA caps so that they avoid activating the MDA5 sensor (Züst et al., 2011). Vertebrate tRNAs also have specific modifications at certain positions in their structures that allow innate immune sensors to discriminate

Figure 5. Aberrant Innate Immune Responses in *Adar1*$^{-/-}$; *p53*$^{-/-}$ Double-Mutant MEFs Are Suppressed by Expression of ADAR Proteins or IU-dsRNA

(A) Expression levels for an array of five ISGs measured in nutrient-starved (72 hr) *Adar1*$^{-/-}$; *p53*$^{-/-}$ MEF cultures following stable knockin of GFP (control) or ADAR proteins. For each gene, values are expressed relative to *p53*$^{-/-}$ (one on y axis, dashed lines) and normalized to the housekeeping gene *ActB*. Error bars, SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus *p53*$^{-/-}$; $^{††}p < 0.01$; $^{†††}p < 0.001$ versus *Adar1*$^{-/-}$; *p53*$^{-/-}$.

(B) Expression levels for an array of 5 ISGs following transfection of *Adar1*$^{-/-}$; *p53*$^{-/-}$ MEF cultures with 0–500 ng *Fluc* mRNA after 12 hr. For each gene, values are expressed relative to mock transfection (0 ng) at 12 hr and normalized to the housekeeping gene *ActB*. Error bars, SEM.

(C) Expression levels for an array of five ISGs following transfection of *Adar1*$^{-/-}$; *p53*$^{-/-}$ MEF cultures with control dsRNA (C-dsRNA) or IU-dsRNA after 12 hr. For each gene, values are expressed relative to that seen after 6 hr in cells transfected with control dsRNA (C-dsRNA) (one on y axis, dashed lines) and normalized to the housekeeping gene *ActB*. Error bars, SEM. $^{*}p < 0.05$; $^{***}p < 0.001$.

(D) Expression levels for an array of five ISGs and one housekeeping gene following transfection of *Adar1*$^{-/-}$; *p53*$^{-/-}$ MEF cultures with 500 ng *Fluc* mRNA (control), with either control dsRNA (C-dsRNA) or IU-dsRNA. For each gene, values are expressed relative to expression after 6 hr in cells transfected with control dsRNA (C-dsRNA) (one on y axis, dashed lines) and normalized to the housekeeping gene *ActB*.

Error bars, SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control.

between them and bacterial tRNAs (Gehrig et al., 2012). Modifications that occur naturally in host RNAs also prevent activation of other innate immune sensors such as PKR and Toll sensors (Nallagatla et al., 2008). Because transfection of I-U dsRNA blocks the aberrant immune activation in *Adar1*; *p53* MEFs, it is most likely that the aberrant immune activation is due to an endogenous dsRNA that activates RLRs. We cannot exclude the possibility that a site-specific editing event contributes, but, if the *Adar1* mutant aberrant immune induction was due to loss of editing in a transcript encoding an innate immune protein or any other indirect effect, then inhibiting RLRs at the level of RNA interaction would be unlikely to rescue.

In the case of I-U dsRNA inhibition of RLRs, the significant effect of sequential I-U wobble base pairs bending and destabilizing dsRNA (McLaughlin and Keegan, 2014) makes it possible to envisage why the sensors are able to detect edited dsRNA (Figure S4). I-U dsRNA binds the RLRs competitively with activating dsRNA (Vitali and Scadden, 2010), probably making grossly similar contacts to the helicase domains and carboxy-terminal domains. RLRs are suitable to assess dsRNA structure because they surround the dsRNA using multiple protein domains to make many contacts to both strands (Kowalinski et al., 2011; Luo et al., 2011). The Hel2i domain, in particular, could have a role in scanning the dsRNA minor groove to detect I-U base pairs and other imperfections (Figures S4C and S4D), possibly leading to alterations in dynamic RLR domain rearrangements required for signaling. Natural RNA duplexes formed by repetitive elements in transcripts will usually be imperfectly paired, and RNA editing will further reduce helical regularity. Formation of regular filaments by RLRs on dsRNA facilitates formation of CARD domain complexes activating MAVS signaling (Kowalinski et al., 2011; Peisley et al., 2014). RLR signaling may be exquisitely sensitive to mismatches and RNA-editing events present in RNA duplexes that indicate the dsRNA is not a direct product of virus replication.

In *Adar1*; *p53* mutant MEFs, the catalytically inactive ADAR1 protein is the least effective at reducing elevated ISG transcripts though it is still RNA binding competent, demonstrating that RNA-editing activity is required. Catalytically active ADAR1 proteins and even a cytoplasmically mislocalized ADAR2 mutant protein (Wong et al., 2003) reduce the aberrantly elevated ISG transcript levels. This emphasizes the role of RNA-editing activity, argues against a dominant role for ADAR1-specific protein-protein or protein-RNA interactions, and suggests that promiscuous editing by ADARs is involved rather than a highly ADAR1-specific editing event. No ADAR1 protein fully suppressed ISG transcripts to levels observed in the $p53^{-/-}$ mutant, but the reintroduced ADAR1 proteins are not expressed under the control of the IFN-inducible *Adar1* promoter itself. Because transfection of general dsRNA oligonucleotides containing inosine-uracil base pairs significantly reduces elevated ISG levels in *Adar1*; *p53* MEFs, the importance of inosine in RNA in preventing the innate immune response is supported in two distinct ways.

Several of the AGS mutations alter residues on the surface of the deaminase domain close to where RNA is predicted to bind (Rice et al., 2012), and it is possible that the *D1113H* mutation, which affects editing activity the least, alters ADAR1 function in other ways such as by perturbing protein and/or RNA interactions required for suppression of innate immunity. In addition to the catalytically active ADAR1 and ADAR2 proteins, two other vertebrate ADARs lack catalytic deaminase activity (for review, see Heale and O'Connell, 2009). Therefore, editing-independent ADAR roles are maintained and could involve protein interactions on dsRNA or sequestration of dsRNA.

While this manuscript was under review, Wang and colleagues showed a substantially stronger editing-independent role of ADAR1 in decreasing innate immune responses in HEK293 (Yang et al., 2014). We also observe some editing-independent rescue, and we suspect that the difference is due to our use of *Adar1*; *p53* MEFs completely lacking ADAR1 and their use of HEK293 cells.

We do not know why *Adar1*; *Mavs* newborn pups die within a day of birth, but preliminary data on cytokine expression in blood from these pups implicate some residual inflammatory effects. Activated RLRs can bypass MAVS and induce inflammasome responses via an alternative pathway (Lucioli et al., 2013). Cell death is a very prominent effect in the *Adar1* mutant, and possible MAVS-bypass signaling to cell death pathways in some cells in *Adar1*; *Mavs* newborn pups also remains to be investigated. Many important autoimmune diseases show transcriptional signatures of elevated ISG expression. Our findings suggest, however, that preventing the aberrant systemic IFN response may not always be sufficient to treat these diseases. When aberrant RLR signaling is activated, the key defects may be cell autonomous. Correcting them will require a much more detailed understanding of intracellular nucleic acid sensing. When the important cellular modified nucleic acids that modulate innate immune responses have been defined, they may point the way to new therapeutic approaches to autoimmune and neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Additional methods are online in the Supplemental Information.

Mouse Genetics

Adar1$^{\Delta 2\text{-}13}$ mice in the C57Bl6N background were obtained from M. Higuchi and P. Seeburg (strain C192) (Hartner et al., 2004). These mice were crossed to the IFN receptor subunit mutant (*Ifnar1*) in the 129 strain background (B&H Animal Suppliers). Two sublines breed *Adar1*$^{-/+}$ in the *Ifnar1*$^{-/-}$ background, and crosses of these mice generate *Adar1*$^{-/-}$; *Ifnar1*$^{-/-}$ embryos. Two further sublines generate heterozygous *Adar1*$^{-/+}$ animals with the mixed B6/129 strain background, and crosses of these generate control *Adar1*$^{-/-}$ embryos without the *Ifnar1* mutation. The *CARDIF*$^{-/-}$ (*MAVS*, *IPS-1*, or *VISA*) mutant line generated by the Tschopp group in Lausanne was obtained from Caetano Reis e Sousa in London. It is homozygous-viable null mutant in the C57B6J background. All experiments on mice were performed in accordance with Edinburgh University rules and National Guidelines on animal experimentation.

Knockin of ADAR cDNA in MEFs

cDNAs encoding ADAR proteins were subcloned into the PiggyBac expression system (System Biosciences) and transfected into *Adar1*$^{-/-}$; *p53*$^{-/-}$ MEFs using LyoVec (InvivoGen). Cells were grown for 2 weeks, and those with successful integration were isolated by fluorescence-activated cell sorting (GFP positive).

Mouse Immune Array

The TaqMan Array Mouse Immune Panel (CN: 4367786; Life Technologies) was used for quantitative gene expression analysis of the immune response signatures in E11.5 whole embryos. Total RNA was extracted and purified,

and cDNA was generated as described above. The Taqman array card was loaded with the first strand cDNA sample and Taqman Universal PCR Master Mix II UNG (Life Technologies) according to the manufacturer's protocol. The amplification was performed on an upgraded version of the Applied Biosystems 7900HT Fast Real-Time PCR System, and the analysis of the data was performed as described above.

ACCESSION NUMBERS

The GEO Accession number for the mouse sequence data from this project is GSE62917.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.041.

AUTHOR CONTRIBUTIONS

N.M.M. performed experiments and assisted with writing the manuscript; S.M.G. performed experiments and assisted with writing the manuscript; R.Y. performed bioinformatic analyses of mouse sequences; S.C. performed experiments; J.B. performed genotyping of mouse strains; D.R. performed genotyping of mouse strains; C.N. performed bioinformatic analyses of mouse sequences; C.V. performed experiments; C.P.P. assisted with bioinformatic analyses of mouse sequences; P.J.M. performed RNA-protein structural analyses, M.F.J. designed experiments; J.D. advised on innate immunity and mouse genetics; I.R.A. performed bioinformatic analyses of repeats; A.D.J.S. designed experiments and provided reagents; M.Ö. assisted with writing the manuscript; L.P.K. performed experiments, designed experiments, and wrote the manuscript; and M.A.O. designed experiments and wrote the manuscript.

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